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Regulated Expression of pH Sensing G Protein-Coupled Receptor-68 Identified through Chemical Biology Defines a New Drug Target for Ischemic Heart Disease

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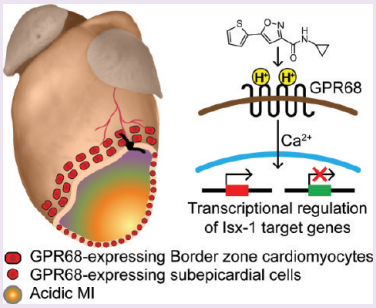
Supporting Information

ABSTRACT: Chemical biology promises discovery of new and unexpected mechanistic pathways, protein functions and disease targets. Here, we probed the mechanism-of-action and protein targets of 3,5-disubstituted isoxazoles (Isx), cardiomyogenic small molecules that target Notch-activated epicardium-derived cells (NECs) *in vivo* and promote functional recovery after myocardial infarction (MI). Mechanistic studies in NECs led to an Isx-activated G_q protein-coupled receptor (G_qPCR) hypothesis tested in a cell-based functional target screen for GPCRs regulated by Isx. This screen identified one agonist hit, the extracellular proton/pH-sensing GPCR GPR68, confirmed through genetic gain- and loss-of-function. Overlooked until now, GPR68 expression and localization were highly regulated in early post-natal and adult post-infarct mouse heart, where GPR68-expressing cells accumulated subepicardially. Remarkably, GPR68-expressing cardiomyocytes established a proton-sensing cellular "buffer zone" surrounding the MI. Isx pharmacologically regulated gene expression (mRNAs and miRs) in this GPR68-enriched border zone, driving cardiomyogenic and pro-survival transcriptional programs *in vivo*. In conclusion, we tracked a (micromolar) bioactive small molecule's mechanism-of-action to a candidate target protein, GPR68, and validated this target as a previously unrecognized regulator of myocardial cellular responses to tissue acidosis, setting the stage for future (nanomolar) target-based drug lead discovery.



Chemical biology promises discovery of new and unexpected functions for cells, genes, proteins and biochemical pathways using small molecule probes that are unbiased with regard to mechanistic preconceptions. Treating bioactive small molecules as mechanistic "black boxes" heightens their discovery promise. Yet, despite advances in chemical informatics, proteomics and affinity chromatography technologies, target identification (target ID) remains a major bottleneck in chemical biology science.[1,2] Successful target ID is particularly important if the goal is drug discovery/development because identifying synthetic small molecule protein targets or receptors allows structure–activity relationship (SAR) studies and pharmacological optimization, moving forward, to be based on direct binding assays or target biochemistry rather than downstream cellular events like reporter gene assays. Target ID is a crucial intermediary and enabling step for advancing primary micromolar hits, which are useful as chemical probes but unlikely to become drugs, toward "druggable" nanomolar leads recovered from chemical structure-focused and target-based secondary screens (Supplemental Figure 1a).

Our stem cell-modulator (gene-activating) small molecules remain mechanistic black boxes.[3] Among our most promising drug-like hits is a family of 3,5-disubstituted isoxazole small molecules (Isx) with differentiating function in hippocampal neural progenitors,[4] malignant glioma[5] and pancreatic β[6] as well as P19CL6 embryonal carcinoma cells.[7] Most recently, despite requiring micromolar concentrations for function in cell-based assays and nonoptimal pharmacokinetics properties, we confirmed Isx efficacy *in vivo* in mice. Delivered as a once daily intraperitoneal (ip) injection, Isx activated distinctive muscle transcriptional programs in Notch-activated epicardium-derived cells (NECs), a rare endogenous adult cardiac progenitor-like cell population.[7−9] Moreover, Isx improved ventricular function in the early phase (1st week) after myocardial infarction (MI).[9] Discovering Isx's mode-of-action and target is the critical next step for advancing this promising small molecule toward drug discovery.

Myocardial acidosis is a central pathophysiological feature of ischemic heart disease. Occluding a major coronary vessel gives rise to focal infarction with cardiomyocyte death in a core region and partially reversible ischemic injury at the infarct's leading edge (the border zone or BZ). Salvaging BZ cardiomyocytes is the fundamental goal of early invasive

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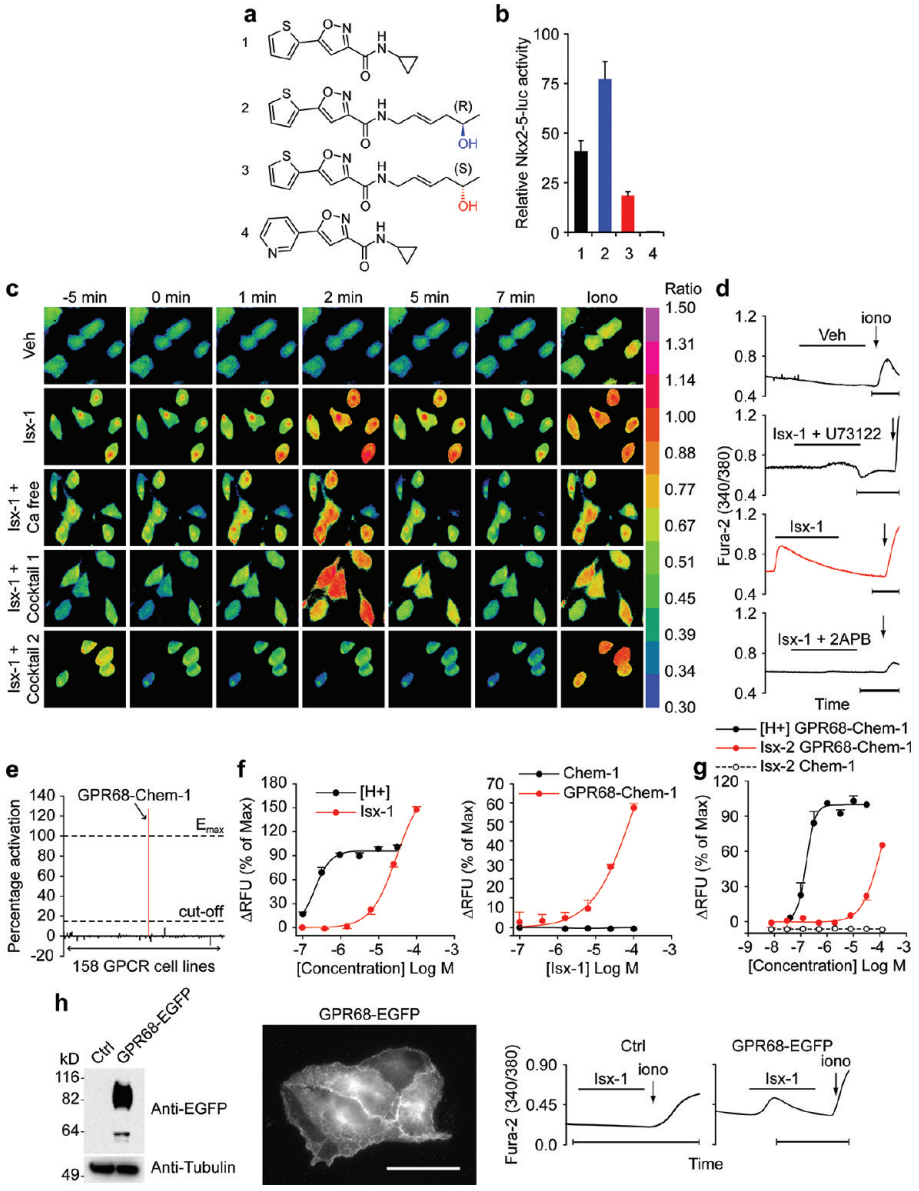

Figure 1. Isx-triggered endoplasmic reticulum Ca^{2+} release and GPCR screen that identified candidate Isx target GPR68 and independent validation. (a) Chemical structures of Isx compounds used in this study: lead-cyclopropyl compound (Isx-1), chiral (*R*)-homoallylic alcohol (Isx-2), chiral (*S*)-homoallylic alcohol (Isx-3) and the non-active pyridine analogue (Isx-4). (b) Differential activity of Isx compounds shown in panel a in Nkx2-5-luc-BAC NECs. (c) $[Ca^{2+}]_i$ signaling triggered by Isx-1 in NECs using Fura-2AM ratiometric imaging (Ca free = Ca^{2+}-free media; Cocktail 1 = 10 μM Nifedipine, 100 μM La^{3+} and 10 μM SKF-96365; Cocktail 2 = 100 μM Ryanodine, 30 μM 2-APB and 5 μM U73122). (d) Examples of Fura-2AM ratiometric $[Ca^{2+}]_i$ tracings of NECs treated with vehicle, Isx-1 or Isx-1 plus inhibitors 5 μM U73122 or 30 μM 2-APB (bar = 1 min). (e) Identification of GPR68-Chem-1 cells as unique agonist hit in Fluo-4 Ca^{2+} flux screen of 158 different recombinant GPCR-expressing cell lines (FLIPR TETRA, Millipore GPCR-Profiler) (E_{max} is maximal activation by control ligand (low pH/protons in this case), and "cut-off" is percentage activation criteria for positive hit in screen). (f) Fluo-4 Ca^{2+} flux signaling activated by Isx-1 is dose-responsive, distinctive from activation by low pH (protons, H+) and is absent in parental Chem-1 cells lacking GPR68. (g) Fluo-4 Ca^{2+} flux signaling is also activated by Isx-2 ((*R*)-enantiomer) and is dose-responsive, with an activation curve that is distinctive from that of low pH/protons. (h) Characterization of a GPR68-EGFP fusion protein-expressing HeLa cell line by protein blot (1st panel) and EGFP fluorescence (2nd panel; scale bar = 20 μm); Fura-2AM ratiometric tracing of Isx-1 triggered $[Ca^{2+}]_i$ signaling in GPR68-EGFP fusion protein-expressing (4th panel) but not parental HeLa cells (Ctrl) (3rd panel).

revascularization strategies in acute coronary care. Falling pH is one of the earliest (within seconds) biomarkers of myocardial ischemia and is caused by cardiomyocytes rapidly switching to anaerobic (lactate-producing) metabolism, coupled with decreased circulatory clearance of lactate and other acidic metabolic byproducts. Protons accumulating with tissue acidosis can function as signaling molecules through a family of proton-sensing G protein-coupled receptors (GPCRs) activated by acidic extracellular pH (~6.4−6.8); this receptor family includes G2A, GPR4, TDAG8, and OGR1 (also known as GPR68).[10−13] Myocardium's metabolic properties make it one the most acidic (and acidosis-prone) tissues in the body, yet proton-sensing GPCRs have never been studied in this tissue.[14]

Here, our mechanistic and target ID studies pointed toward a proton-sensing GPCR that, remarkably, was highly regulated in ischemic myocardium, upregulated precisely when and where an acidosis-sensing (and Isx small-molecule targeted) pro-

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survival function would be critically needed. Our results validate the promise of the chemical biology discovery cycle (Supplemental Figure 1a) for identifying new mechanisms and disease targets.

RESULTS AND DISCUSSION

Isx Stereoselectivity Implies a Ligand−Receptor Mechanism in Target Cells. Despite defining several important pharmacological functions in a broad spectrum of progenitor-like cells,[3−6] even *in vivo*,[9] Isx's precise mechanism-of-action has remained elusive. *N*-cyclopropyl-5-(thiophen-2-yl)-isoxazole-3-carboxamide, our top Isx lead (henceforth referred to as "Isx-1"; Figure 1a), robustly activated Nkx2-5-luc-BAC in NECs, increasing luciferase specific activity (per μg protein) ∼40-fold (Figure 1b). In a concerted effort to identify more potent leads, we synthesized a library of Isx analogues (∼100 compounds) based on the structure of Isx-1 through an iterative ligand-based medicinal chemistry approach. Out of this focused library, we identified a new lead containing a chiral (*R*)-homoallylic alcohol amide side chain (Isx-2) (Figure 1a) that was even more effective than Isx-1 in Nkx2-5-luc-BAC NECs, activating the reporter gene ∼75-fold over vehicle (Figure 1b). Realizing that enantiomers can have significantly different affinities for a given biological target,[15] we also synthesized the corresponding (*S*)-enantiomer of this new lead (Isx-3) (Figure 1a) and compared both enantiomers in Nkx2-5-luc-BAC NECs (Figure 1b). Indeed, under identical experimental conditions, Isx-3 had markedly reduced bioactivity compared to that of Isx-2 (Figure 1b). Additionally, from our library we also identified an analogue suitable as a negative control for our assays by demonstrating that substituting a pyridine for the thiophene ring in Isx-1 (Isx-4) (Figure 1a) abolished function (Figure 1b). Thus, otherwise identical stereoisomers (Isx-2 and -3) had substantially different biological activities, providing strong evidence of a very discrete and stereoselective ligand/receptor interaction between Isx and its cognate target protein.

Isx Triggers Endoplasmic Reticulum Ca^{2+} Release Through GPCR Activation. While systematically surveying candidate modes-of-action, we asked whether Isx-1 regulated intracellular Ca^{2+} flux in NECs. Indeed, Isx-1 triggered an intracellular Ca^{2+} transient $[Ca^{2+}]_i$ in NECs that was rapid in upstroke, high in amplitude and short in duration, with $[Ca^{2+}]_i$ quickly returning to baseline (without drug-washout) (Figure 1c and d). Importantly, this intracellular Ca^{2+} transient was distinctive from the signal previously reported in adult hippocampal neural progenitors.[4] We localized the source of this Ca^{2+} release by removing Ca^{2+} from the media (Ca free), adding Ca^{2+} influx inhibitors (cocktail 1: Nifedipine, La^{3+}, and SKF96365) or blockers of Ca^{2+} release from intracellular stores (cocktail 2: U73122, 2-APB, and Ryanodine). Only cocktail 2 blocked the Isx-1 triggered $[Ca^{2+}]_i$ spike in NECs (Figure 1c). More specifically, 2-APB (an inositol 1,4,5-triphosphate receptor antagonist) and U73122 (a G_qPCR signaling inhibitor) each independently blocked the Isx-1 induced $[Ca^{2+}]_i$ spike in NECs (Figure 1d), mapping the origin of Isx-1's signal to endoplasmic reticulum Ca^{2+} release through GPCR activation. These results strongly suggested an Isx-GPCR mechanistic pathway.

Functional Target Screen for GPCRs Regulated by Isx Identifies GPR68. We tested the Isx-GPCR hypothesis by screening a library of recombinant GPCR-overexpressing cell lines in a functional assay based on Fluo-4 Ca^{2+} flux ($FLIPR^{TETRA}$; Millipore GPCR-Profiler). Millipore's GPCR-Profiler screen, covering all receptor classes and ligand families, simultaneously evaluated 158 cell lines for Isx-1 mediated agonist, antagonist, and allosteric modulator Ca^{2+} signaling activity. The Isx-1 agonist screen yielded a single hit: GPR68-Chem-1 cells engineered to overexpress human GPR68 (a G_q protein-coupled receptor) in rat Chem-1 cells (which express $G_{\alpha 15}$, a promiscuous G protein that enhances GPCR coupling to downstream Ca^{2+} signaling pathways) (Figure 1e). The kinetics of Isx-1 mediated Ca^{2+} signaling in GPR68-Chem-1 cells was most consistent with a direct agonist rather than a downstream effect. Importantly, Isx-1 is a neutral molecule (Figure 1a), so its effects were not mediated through pH changes. We did a number of secondary experiments to confirm and validate this screen hit. For example, we demonstrated dose-responsive activation of GPR68-Chem-1 cell Ca^{2+} signaling by Isx-1 (compared side-by-side with protons $[H^+]$, the control "ligand" for this pH-sensing GPCR) (Figure 1f). In addition, we demonstrated that Isx-1 triggered Ca^{2+} signaling in GPR68-Chem-1 cells, but not in parental Chem-1 cells (lacking overexpressed GPR68) (Figure 1f). We independently confirmed that a second structurally distinct Isx analogue, Isx-2, the chiral (*R*)-homoallylic alcohol amide (Figure 1a), activated Ca^{2+} signaling in this cell line (Figure 1g). Like Isx-1, Isx-2's activity was dose-responsive in GPR68-Chem-1 but not parental Chem-1 cells (Figure 1g). Thus, our cell-based screen for GPCRs regulated by Isx identified a single hit, GPR68, an unexpected candidate target with no known function in the heart.

Genetic Gain-of-Function Validates Isx Candidate Target. We confirmed and extended the Millipore GPCR-Profiler and follow-up studies by independently establishing our own HeLa cell line that overexpressed a GPR68-EGFP fusion protein,[11] which localized predominantly to the plasma membrane (Figure 1h). Isx-1 activated Ca^{2+} signaling (increased $[Ca^{2+}]_i$) in GPR68-EGFP HeLa but not in control (parental) cells (Figure 1h). Notably, the morphology and kinetics of the Isx-1 triggered $[Ca^{2+}]_i$ release in GPR68-EGFP HeLa cells (Figure 1h) was indistinguishable from the native Isx-1 triggered $[Ca^{2+}]_i$ signal in NECs (Figure 1d). These gain-of-function experiments in HeLa cells independently corroborated the results of the Millipore GPCR-Profiler screen.

GPR68-Expressing Cells Localize to Postnatal and Injury-Activated Subepicardium. Regulated expression of genes in developmental or pathophysiological time and space provides evidence of that gene product's functional importance. GPR68 expression in myocardium, one of the most acidosis-prone tissues in the body,[16] had not been previously characterized. As a starting point, we evaluated GPR68 expression in mice during the first 2 weeks of postnatal life, a period of rapid structural and physiological maturation, using an antibody validated in GPCR immunohistochemistry (IHC). We observed widespread GPR68 staining in newborn mouse myocardium, which is composed almost entirely of cardiomyocytes, at days 1 and 3 of postnatal life (Figure 2a, upper panels). However, between postnatal days 7 and 14, mouse heart GPR68 staining increasingly concentrated in subepicardium (Figure 2a, lower panels). Thus, GPR68-expressing cells progressively localized to neonatal mouse subepicardium.

MI "activates" subepicardium, amplifying and recruiting subepicardial cells like NECs into tissue repair pathways.[7,17] Therefore, to provide evidence for GPR68's possible role in epicardium-mediated myocardial injury repair, we studied GPR68-expressing cells in this tissue. Indeed, MI markedly

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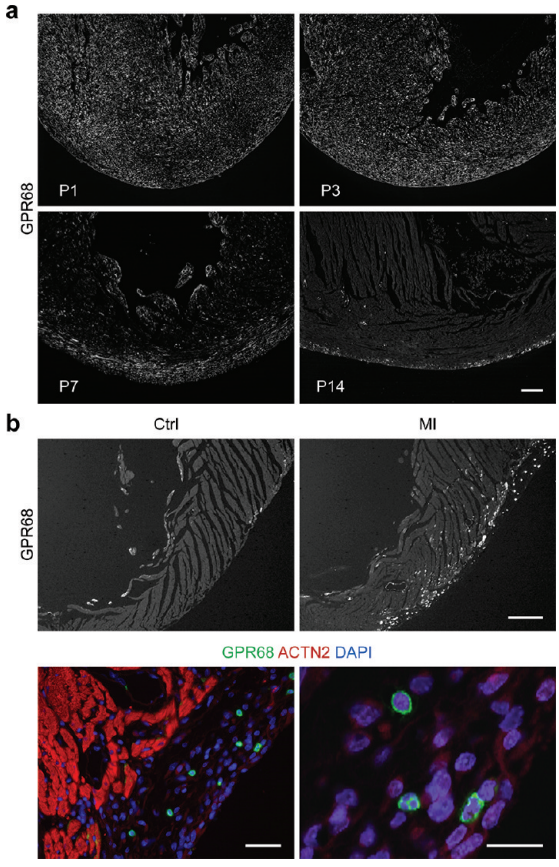

Figure 2. Progressive accumulation of GPR68-expressing cells in neonatal and injury-activated subepicardium. (a) IHC localization of GPR68-expressing cells in neonatal mouse heart at postnatal days 1, 3, 7 and 14 (from upper left to lower right panels) (scale bar = 50 μm). (b) Accumulation of GPR68-expressing cells in injury-activated subepicardium of adult mouse heart (upper right-hand panel) compared to control/uninjured heart (upper left-hand panel) by IHC (scale bar =200 μm); higher magnification images of GPR68-expressing cells (green) in the injury-activated subepicardial region of day 7 post-infarct adult mouse heart, co-stained with ACTN2 (red) and DAPI (blue) (scale = 20 μm).

increased the number of GPR68-expressing cells in the injury-expanded subepicardium (Figure 2b, right upper panel). In control (uninjured) hearts, we detected rare GPR68-expressing cells, and these were localized mostly to endocardium (Figure 2b, left upper panel). After MI, we observed numerous distinctive small round cells expressing high levels of GPR68 throughout the subepicardium (Figure 2b, lower panels). Thus, GPR68-expressing cells localized to injury-activated subepicardium, implicating GPR68, the proton-sensing and candidate Isx receptor, in cardiac repair pathways that involve epicardium-derived cells.

A GPR68-Expressing Buffer Zone Encapsulates the Ischemic/Acidic Infarct. The adult mouse heart's response to experimental left anterior coronary artery ligation MI is complex and dynamic. We expanded our analysis of GPR68 in MI injured myocardium by dividing post-infarct tissue into three discrete zones, by convention: the ischemic (IZ), border (BZ), and remote zones (RZ) (Figure 3a). When compared to normal myocardium, 1 week after injury, all three zones had increased Gpr68 mRNA levels, more than 5-fold in IZ and BZ, measured by quantitative polymerase chain reaction (QPCR) (Figure 3b, left-hand graph). We reinforced this result by examining NECs from post-infarct or control hearts. Indeed, compared with uninjured controls, NECs from day 7 post-infarct hearts had increased Gpr68 mRNA levels by QPCR (Figure 3b, right-hand graph). Thus, at the gene expression level, MI increased Gpr68 in BZ and, more specifically, in NEC heart-repair cells.[18]

Returning to GPR68 IHC, we corroborated these QPCR data by localizing GPR68 in post-MI adult mouse heart, comparing RZ and BZ. At 7 days post-infarct, compared with RZ (Figure 3c, upper panels), BZ had markedly increased GPR68 expression (Figure 3c, remaining panels). The majority of GPR68-expressing BZ cells appeared to be cardiomyocytes, confirmed by confocal microscopy (see below). This unique distribution of GPR68-expressing cells in the BZ and injury-activated epicardium established a proton-sensing "cellular buffer zone" at the boundary of spared myocardium, three-dimensionally shielding viable myocardium from the ischemic/acidic infarct zone (see model, Supplemental Figure 1b).

Isx Drives Cardiomyocyte-Sparing Gene Programs in GPR68-Expressing BZ *in Vivo*. As a myocardial region enriched with GPR68-expressing cells, MI BZ should be highly responsive to Isx's *in vivo* pharmacological actions. We focused here on pro-apoptotic miR-15, a key regulator of ischemic cardiomyocyte death in the MI BZ.[19] We evaluated miR-15a and miR-15b levels in RNA isolated from RZ and BZ tissues of a cohort of mice, using QPCR to measure transcript levels. We compared 4 treatment arms: sham operation versus MI and Isx-1 versus vehicle treatment for 7 days, starting on postoperative day 1 (Figure 3d). After 7 days, Isx-1 upregulated miRs-15a and -15b in RZ, in sham and MI mice (Figure 3d, left-hand panel). In contrast, in MI BZ, Isx-1 completely blocked the normally observed induction of miRs-15a and -15b.[19] Correspondingly, Isx-1 negatively regulated pro-apoptotic miR-15b and reciprocally increased myogenic miRs-1 and -133a in a time- (Figure 3e, left-hand panel) and dose-dependent manner (Figure 3e, right-hand panel). These data demonstrated that Isx pharmacologically regulated miR gene expression, in a direction that favored cardiomyocyte survival, in the proton-sensing "cellular buffer zone" surrounding the infarct *in vivo*.

Next, we focused on Isx-1's cardiomyogenic pharmacological signal in MI BZ *in vivo*. Isx-1 therapy for 7 days post-infarct increased muscle [α-actinin (Actn2), myomesin-1 (Myom1), Nkx2-5, brain natriuretic peptide (Nppb) and troponin C (Tnnc1)] and, conversely, decreased collagen (Col1a1, Col1a2) transcript levels *in vivo* (Figure 3f, left-hand panel). As controls, we observed unchanged connective tissue growth factor (Ctgf) or increased elastin (Eln) extracellular matrix (ECM) transcript levels in Isx-1 treated MI BZ tissue *in vivo* (Figure 3f, left-hand panel). Furthermore, we confirmed these *in vivo* results in cultured NECs (Figure 3f, right-hand panel). With the exception of elastin, the direction of gene expression changes induced by Isx-1 in NECs mirrored the QPCR results observed in MI BZ (Figure 3f, right and left-hand panels). Confirming that Isx-1 induced mRNA changes indeed translated into changes in protein levels, Isx-1 concordantly upregulated CTGF protein and mRNA in cultured NECs (Figure 3f, right-hand panel and Supplemental Figure 2). Taken together, these data demonstrated that Isx-1 indeed targeted GPR68-expressing MI BZ *in vivo*, driving pro-survival and cardiomyogenic transcriptional programs, and that cultured NECs, for the most part, modeled these Isx-1 mediated transcriptional effects *in vitro*.

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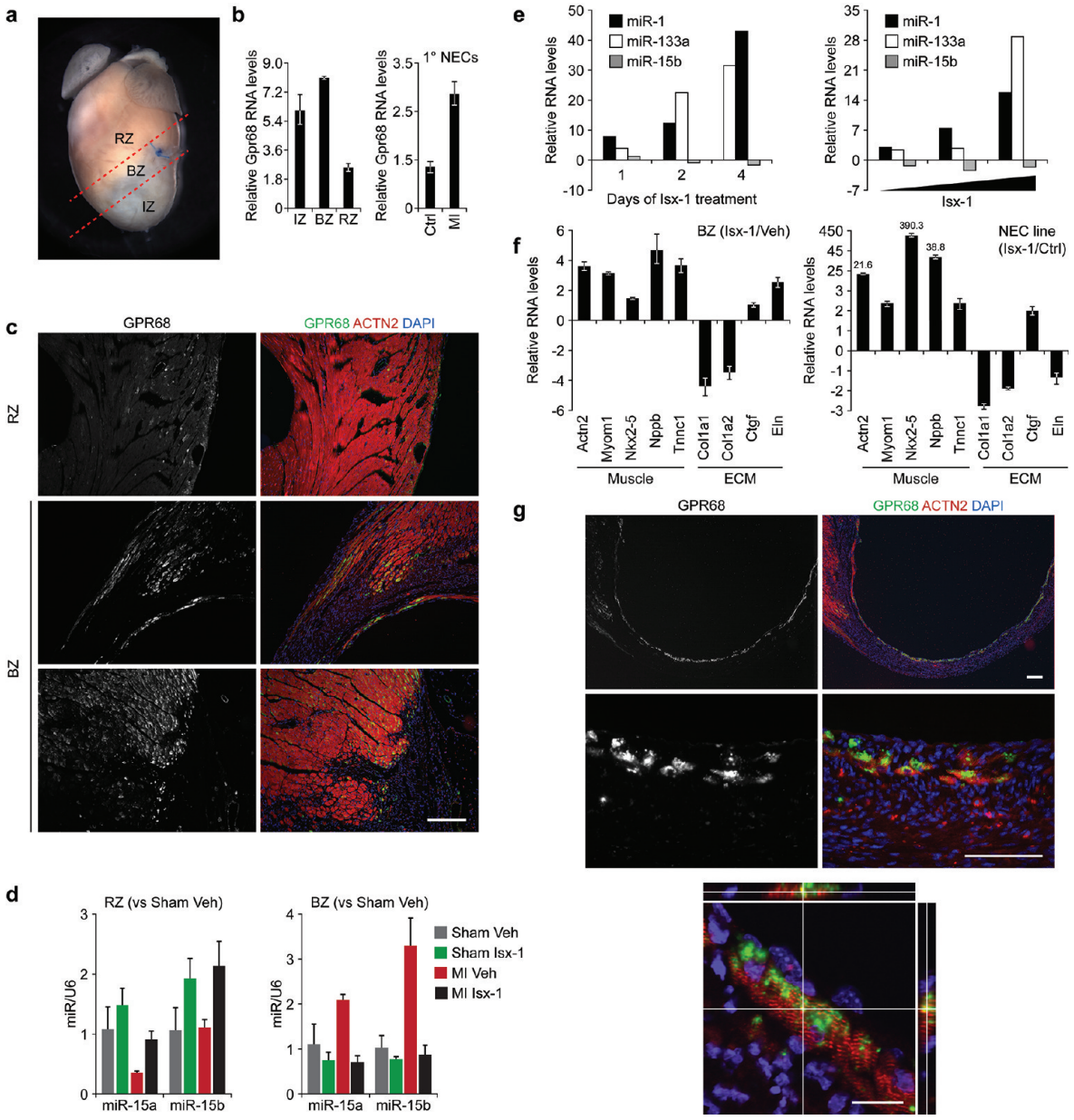

Figure 3. Isx targets the GPR68-enriched MI BZ and drives cardiomyocyte-sparing gene programs. (a) Post-MI adult mouse heart demonstrating conventional subdivision of heart into remote zone (RZ), border zone (BZ), which includes the suture on the left anterior descending coronary artery and infarct zone (IZ). (b) Upregulation of Gpr68 mRNA by QPCR in IZ, BZ and RZ of the adult mouse heart at day 7 post-MI (compared to sham operation) (left-hand panel) and upregulation of Gpr68 mRNA in primary NECs at day 7 post-MI (right-hand panel). (c) IHC localization of GPR68 in the RZ (top panels) and BZ (middle and bottom panels) in MI injured adult mouse heart (left-hand panels, GPR68 alone; right-hand panels, GPR68 (green) co-stained with ACTN2 (red) and DAPI (blue)) (scale bar = 50 μm). (d) Regulation of miRs-15a and -15b by QPCR in RZ (left-hand graph) or BZ (right-hand graph) tissues of 4 mouse cohorts (sham or MI treated with Isx-1 or vehicle; sham-vehicle n = 2, sham-Isx-1 n = 3, MI-vehicle n = 3, and MI-Isx-1 n = 5) at day 7. (e) Time- (left-hand panel) and dose-dependent (right-hand panel) regulation of myogenic miRs-1 and −-133a and pro-apoptotic miR-15b in Isx-1 treated NECs. (f) Reciprocal regulation of cardiomyogenic (Actn2, Myom1, Nkx2-5, Nppb and Tnnc1) and extracellular matrix remodeling/fibrosis (Col1a1 and Col1a2, Ctgf and Eln) genes by QPCR in BZ tissue RNA from Isx-1 versus vehicle-treated MI mice (n = 2 mice per group) (left panel); expression analysis of same gene set *in vitro* in 4-day Isx-1 treated versus untreated NECs (right panel). (g) IHC localization of GPR68-expressing cardiomyocytes in the infarct zone in MI adult mouse heart (left-hand panels, GPR68 alone; right-hand panels, GPR68 (green) co-stained with ACTN2 (red) and DAPI (blue)) at low (top panels) and high (bottom panels) magnification (scale bar = 200 μm) and confocal microscopy of a single GPR68-expressing cardiomyocyte (bottom panel, scale bar = 20 μm).

MI-Spared Cardiomyocytes Express High Levels of GPR68. Our data thus far suggested that GPR68, normally expressed at only very low levels but upregulated following injury, specifically in BZ, transduced pro-survival and cardiomyogenic signals in the heart. We obtained additional evidence for GPR68's cardioprotective function by IHC in MI mice (Figure 3g). We observed a thin rim of surviving cardiomyocytes near the endocardial surface of a large transmural apical MI (Figure 3g, upper panels). When viewed at higher magnification (Figure 3g, lower panels), it became evident that each and every surviving cardiomyocyte in this region expressed high levels of GPR68. These expression data

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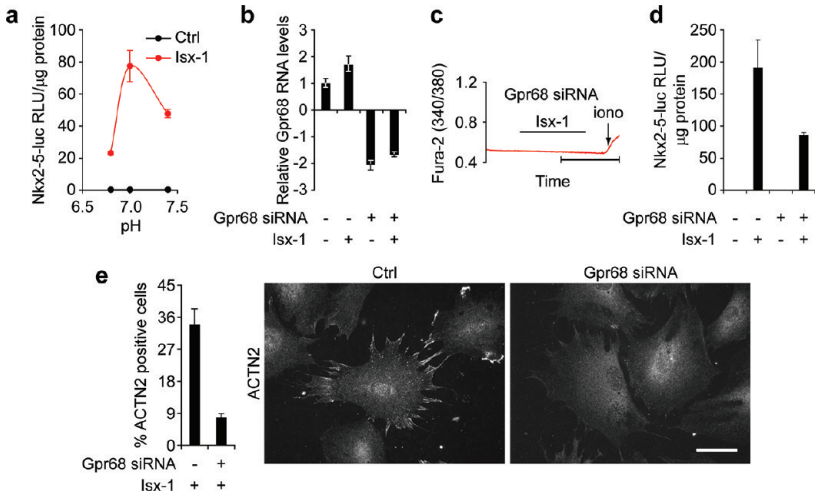

Figure 4. GPR68 is required for Isx's cardiomyogenic function in NECs. (a) Activation of Nkx2-5-luc-BAC by Isx-1 in NECs is regulated by pH (maximal activity at pH ≈ 7.0), but low pH/protons alone (Ctrl) does not activate this reporter gene. (b) siRNA-mediated Gpr68 mRNA knockdown by QPCR in NECs. siRNA-mediated Gpr68 mRNA knockdown abolished Isx-1 triggered Ca^{2+} signaling by Fura-2AM ratiometric tracing (c), attenuated Isx-1 mediated Nkx2-5-luc-BAC reporter gene activation (d), and decreased Isx-1 induced biosynthesis and assembly of α-actinin pre-sarcomeres in NECs shown graphically (left-hand graph) and in representative Ctrl or Gpr68 siRNA ACTN2 IHC images (scale bar = 50 μm) (e).

suggested that GPR68 conferred a survival advantage to cardiomyocytes at the MI frontier. We used confocal microscopy to confirm that GPR68 localized to cardiomyocytes (Figure 3g, bottom panel). Thus, MI-spared cardiomyocytes expressed high levels of GPR68, providing a mechanistic hypothesis for how Isx-1 might have regulated cardiac function after MI *in vivo*.[9]

GPR68 Is Required for Isx's Cardiomyogenic Function in NECs. We used genetic loss-of-function in NECs to confirm GPR68's role in Isx-1 triggered Ca^{2+} signaling, transcriptional activation, and cardiomyogenic differentiation. First, moderate extracellular acidosis (pH 7.0) mimicked Isx-1's effects on NEC morphology (flattening), suggesting that protons and Isx were acting through a convergent mechanistic pathway (data not shown). Moreover, extracellular acidosis (pH 7.0) sensitized NECs to Isx-1 mediated Nkx2-5-luc-BAC activation, although, importantly, low pH alone did not transduce sufficient signal to activate the reporter gene in these cells (Figure 4a). We confirmed GPR68's role in normal Isx function using small interfering RNAs (siRNA), which knocked down Gpr68 mRNA levels by ∼50% in Nkx2-5-luc-BAC reporter NECs, independent of Isx (Figure 4b). GPR68 knockdown by siRNA completely abolished the Isx-triggered Ca^{2+} spike in NECs (Figure 4c) and, further downstream, strongly attenuated (by almost two-thirds) the Isx-1 induced transactivation of the Nkx2-5-luc-BAC in reporter NECs (Figure 4d). Finally, we studied the effects of Gpr68 on Isx-1 mediated cardiomyogenic differentiation in NECs by evaluating the biosynthesis and assembly of α-actinin pre-sarcomeres (Figure 4e). Specific downregulation of GPR68 by siRNA dramatically inhibited Isx-1's cardiomyogenic differentiation activity in NECs. Taken together, these data demonstrate that GPR68 is an important mediator of Isx-1 triggered Ca^{2+} fluxes, downstream transcriptional regulatory circuits and differentiation programs in NECs.

Conclusion. Identifying the mechanism-of-action and targets of bioactive synthetic small molecules is a crucial bottleneck for advancing primary (micromolar) hits useful as chemical probes toward target-based (nanomolar) leads suitable for drug discovery and development. The unbiased nature of primary hits from cell-based "black box" screens can make traversing this chemical biology bottleneck an "aha" moment of surprise and mechanistic clarity. Here, we probed the mechanism-of-action and protein targets of our Isx small-molecule drivers of cardiomyogenic differentiation in NECs and enhancers of post-MI ventricular function in mice.[9] We tested our Isx-GPCR hypothesis in a functional target screen that identified GPR68, a proton-sensing GPCR highly regulated in ischemic myocardium. Conceptually, GPR68 provides an ideal (disease-specific) target for future drugs intended to enhance myocardial cell survival and function in the context of ischemic acidosis. GPR68 joins an elite class of myocardial G_qPCRs that includes angiotensin-II (AT1R), adrenergic (α-1AR), and endothelin-1 (ET1R) receptors.[20] Connecting GPR68 to ischemic heart disease through Isx chemical probes validates chemical biology's promise to discover new and unexpected disease targets.

■ ASSOCIATED CONTENT

Supporting Information

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Burdine, L., and Kodadek, T. (2004) Target identification in chemical genetics: the (often) missing link. *Chem. Biol. 11*, 593−597.
(2) Laggner, C., Kokel, D., Setola, V., Tolia, A., Lin, H., Irwin, J. J., Keiser, M. J., Cheung, C. Y., Minor, D. L., Jr., Roth, B. L., Peterson, R. T., and Shoichet, B. K. (2011) Chemical informatics and target identification in a zebrafish phenotypic screen. *Nat. Chem. Biol. 8*, 144−146.
(3) Sadek, H., Hannack, B., Choe, E., Wang, J., Latif, S., Garry, M. G., Garry, D. J., Longgood, J., Frantz, D. E., Olson, E. N., Hsieh, J., and Schneider, J. W. (2008) Cardiogenic small molecules that enhance myocardial repair by stem cells. *Proc. Natl. Acad. Sci. U.S.A. 105*, 6063−6068.
(4) Schneider, J. W., Gao, Z., Li, S., Farooqi, M., Tang, T. S., Bezprozvanny, I., Frantz, D. E., and Hsieh, J. (2008) Small-molecule activation of neuronal cell fate. *Nat. Chem. Biol. 4*, 408−410.
(5) Zhang, L., Li, P., Hsu, T., Aguilar, H. R., Frantz, D. E., Schneider, J. W., Bachoo, R. M., and Hsieh, J. (2011) Small-molecule blocks malignant astrocyte proliferation and induces neuronal gene expression. *Differentiation 81*, 233−242.
(6) Dioum, E. M., Osborne, J. K., Goetsch, S., Russell, J., Schneider, J. W., and Cobb, M. H. (2011) A small molecule differentiation inducer increases insulin production by pancreatic beta cells. *Proc. Natl. Acad. Sci. U.S.A. 108*, 20713−20718.
(7) Russell, J. L., Goetsch, S. C., Gaiano, N. R., Hill, J. A., Olson, E. N., and Schneider, J. W. (2011) A dynamic notch injury response activates epicardium and contributes to fibrosis repair. *Circ. Res. 108*, 51−59.
(8) Rentschler, S., and Epstein, J. A. (2011) Kicking the epicardium up a notch. *Circ. Res. 108*, 6−8.
(9) Russell, J. L., Goetsch, S. C., Aguilar, H., Frantz, D. E., and Schneider, J. W. (2012) Targeting native adult heart progenitors with cardiogenic small-molecules. *ACS Chem. Biol.*, DOI: 10.1021/cb200525q.
(10) Ludwig, M. G., Vanek, M., Guerini, D., Gasser, J. A., Jones, C. E., Junker, U., Hofstetter, H., Wolf, R. M., and Seuwen, K. (2003) Proton-sensing G-protein-coupled receptors. *Nature 425*, 93−98.
(11) Radu, C. G., Nijagal, A., McLaughlin, J., Wang, L., and Witte, O. N. (2005) Differential proton sensitivity of related G protein-coupled receptors T cell death-associated gene 8 and G2A expressed in immune cells. *Proc. Natl. Acad. Sci. U.S.A. 102*, 1632−1637.
(12) Tomura, H., Mogi, C., Sato, K., and Okajima, F. (2005) Proton-sensing and lysolipid-sensitive G-protein-coupled receptors: a novel type of multi-functional receptors. *Cell. Signalling 17*, 1466−1476.
(13) Seuwen, K., Ludwig, M. G., and Wolf, R. M. (2006) Receptors for protons or lipid messengers or both? *J. Recept. Signal Transduction Res. 26*, 599−610.
(14) Poole-Wilson, P. A. (1982) Acidosis and contractility of heart muscle. *Ciba Found. Symp. 87*, 58−76.
(15) Klabunde, T., and Hessler, G. (2002) Drug design strategies for targeting G-protein-coupled receptors. *ChemBioChem 3*, 928−944.
(16) Xu, Y., and Casey, G. (1996) Identification of human OGR1, a novel G protein-coupled receptor that maps to chromosome 14. *Genomics 35*, 397−402.
(17) Zhou, B., Honor, L. B., He, H., Ma, Q., Oh, J. H., Butterfield, C., Lin, R. Z., Melero-Martin, J. M., Dolmatova, E., Duffy, H. S., Gise, A., Zhou, P., Hu, Y. W., Wang, G., Zhang, B., Wang, L., Hall, J. L., Moses, M. A., McGowan, F. X., and Pu, W. T. (2011) Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J. Clin. Invest. 121*, 1894−1904.
(18) Russell, J. L., Goetsch, S. C., Gaiano, N. R., Hill, J. A., Olson, E. N., and Schneider, J. W. (2010) A dynamic notch injury response activates epicardium and contributes to fibrosis repair. *Circ. Res. 108*, 51−59.
(19) Hullinger, T. G., Montgomery, R. L., Seto, A. G., Dickinson, B. A., Semus, H. M., Lynch, J. M., Dalby, C. M., Robinson, K., Stack, C., Latimer, P. A., Hare, J. M., Olson, E. N., and van Rooij, E. (2012) Inhibition of miR-15 Protects Against Cardiac Ischemic Injury. *Circ. Res. 110*, 71−81.
(20) Salazar, N. C., Chen, J., and Rockman, H. A. (2007) Cardiac GPCRs: GPCR signaling in healthy and failing hearts. *Biochim. Biophys. Acta 1768*, 1006−1018.
(21) Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods 25*, 402−408.
(22) Luo, X., Shin, D. M., Wang, X., Konieczny, S. F., and Muallem, S. (2005) Aberrant localization of intracellular organelles, Ca2+ signaling, and exocytosis in Mist1 null mice. *J. Biol. Chem. 280*, 12668−12675.